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The Virulence Gene Cluster of *Listeria monocytogenes* Is Also Present in *Listeria ivanovii*, an Animal Pathogen, and *Listeria seeligeri*, a Nonpathogenic Species

EDITH GOUIN, JÉROME MENGAUD, AND PASCALE COSSART*

Unité des Interactions Bactéries-Cellules, Centre National de la Recherche Scientifique URA 1300, Institut Pasteur, Paris 75015, France

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Most known *Listeria monocytogenes* virulence genes cluster within a 9.6-kb chromosomal region. This region is flanked on one end by two uncharacterized open reading frames (ORF A and ORF B) and *ldh*, an ORF presumably encoding the *L. monocytogenes* lactate dehydrogenase (J.-A. Vazquez-Boland, C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart, Infect. Immun. 60:219–230, 1992). We report here that the other end is flanked by *prs*, an ORF homologous to phosphoribosyl PP_i synthetase genes. ORF B and *prs* were detected in all *Listeria* species and thus delimit the virulence region. This virulence gene cluster was detected exclusively in hemolytic *Listeria* species, *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species.

Among the six species of the genus *Listeria*, only two are pathogenic. *Listeria monocytogenes* is pathogenic for humans and animals, and *Listeria ivanovii* is pathogenic for animals (16). *L. monocytogenes* causes severe infections that primarily affect immunocompromised people, pregnant women, and neonates but occasionally also healthy people. It has been recognized as a food pathogen after several listeriosis outbreaks traced to contaminated food (for a review, see reference 11). In animals, *L. monocytogenes* primarily affects sheep and cows and is of some veterinary importance. The incidence of *L. ivanovii* in animal listeriosis is very low (5, 20).

L. monocytogenes is a facultative intracellular bacterium which, because of the development of genetic tools and in vitro models of infection, has recently become one of the best systems approaching the genetic and molecular basis of intracellular parasitism (7). A precise description of the cell infectious process has emerged (for a review, see reference 34). It can be divided into four major steps: entry into the cell, escape from the phagosomal compartment, intracytosolic multiplication, and cell-to-cell spread. This last step involves bacterial movement within the cytoplasm of the infected cell. This movement is mediated by a mechanism involving continuous actin assembly at the rear of the bacterium (for reviews, see references 6 and 36).

Several virulence genes have been identified (for reviews, see references 32 and 34). Apart from genes *inlAB* (12) and *iap* (23) which are, respectively, involved in and associated with entry, all identified virulence genes map to the same chromosomal region around the *hly* gene (29). The *hly* gene encodes a hemolysin, called listeriolysin O (for a review, see reference 7), that is essential for lysis of the phagosomal membrane. This gene is flanked by two operons, the *plcA-prfA* operon and the lecithinase operon. The lecithinase operon contains at least two genes involved in virulence: *actA*, required for actin assembly (9, 22), and *plcB*, which encodes a lecithinase involved in cell-to-cell spread (38). Upstream from *hly*, *plcA* encodes a phosphatidylinositol-specific phospholipase C (2, 25, 27, 31) which may contribute to the lysis of the phagosomal membrane (3). *plcA* is followed by gene *prfA*, which encodes a pleiotropic activator of *plcA*, *hly*, and the lecithinase operon as well as *inlAB* (4, 10, 26, 28).

The nucleotide sequence of the 1.8-kb region lying downstream from *prfA* has now been determined from pLis29 (28), a pUC derivative used previously to determine the sequence of *prfA* (Fig. 1) (GenBank accession no. M92842). It reveals the presence of two open reading frames (ORFs) in opposite orientations to *prfA*. The first of these ORFs is 954 bp long and ends 48 bp downstream from *prfA*. It encodes a protein of 318 amino acids whose deduced amino acid sequence is similar to the sequences of phosphoribosyl PP_i (PRPP) synthetases of *Bacillus subtilis* (30) (77% identity on a 317-amino-acid overlap), *Escherichia coli* (18) (47.1% identity on a 314-amino-acid overlap), and *Salmonella typhimurium* (1) (47.5% identity on a 314-amino-acid overlap) and to human and rat PRPP synthetases (19, 35) (45.8% identity on a 314-amino-acid overlap). We have therefore named this ORF *prs*. On the same DNA strand, 54 bp upstream from *prs*, another 753-bp-long ORF was identified. Sequence analysis suggested that this ORF is incomplete in pLis29. The deduced amino acid sequence of this ORF is homologous to the C terminus of the proteins encoded by *tms*, the gene located just upstream from *prs* in *B. subtilis* (30) (55.2% similarity on a 248-amino-acid overlap), and the *ecourf1* gene of *E. coli* (39) (39.4% similarity on a 249-amino-acid overlap). We have therefore named this second ORF *tms*.

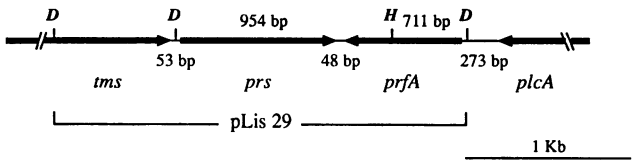

FIG. 1. Physical and genetic map of the region located downstream from *prfA*. The upper line represents the genetic organization of the region. D and H correspond to *Dra*I and *Hin*dIII sites, respectively. The lengths of the genes and of the intergenic regions are indicated. The lower line represents the insert in pLis29 (28).

* Corresponding author. Mailing address: Unité des Interactions Bactéries-Cellules, Institut Pasteur, 28 rue du Dr. Roux, Paris 75015, France. Phone: 33-1-45688841. Fax: 33-1-45688706. Electronic mail address: pcossart@pasteur.fr.

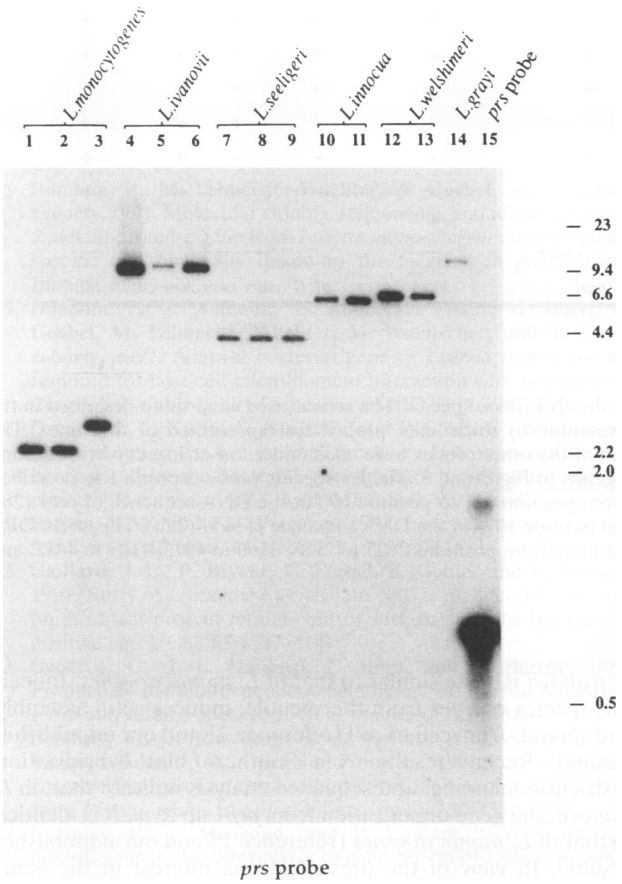

FIG. 2. Southern blot of DNAs from the six *Listeria* species hybridized under high-stringency conditions with a *prs*-specific probe. Lanes: 1, *L. monocytogenes* LO28 (Bof 343); 2, *L. monocytogenes* EGD (BUG 600); 3, *L. monocytogenes* type strain SLCC53^T (BUG 31); 4, *L. ivanovii* type strain CIP7842^T (BUG 496); 5, *L. ivanovii* CLIP257 (BUG 497); 6, *L. ivanovii* SLCC4121 (BUG 497); 6, *L. ivanovii* SLCC4121 (BUG 598); 7, *L. seeligeri* type strain SLCC3954^T (BUG 494); 8, *L. seeligeri* CLIP 9529 (BUG 495); 9, *L. seeligeri* SLCC3503 (BUG 599); 10, *L. innocua* type strain CIP8011^T (BUG 498); 11, *L. innocua* CLIP11262 (BUG 499); 12, *L. welshimeri* SLCC5328 (BUG 501); 13, *L. welshimeri* type strain SLCC5334^T (BUG 502); 14, *L. grayi* type strain CIP76124^T (BUG 503); 15, the *prs*-specific probe. Molecular weight markers in kilobases are indicated on the right. All lanes contain the same amount of DNA except for lane 5 which, from the ethidium bromide staining, contains at least five times less DNA. The *prs*-specific probe is a PCR DNA fragment spanning from position 845 to position 1791 in the DNA sequence of *prs* (GenBank accession no. M92842).

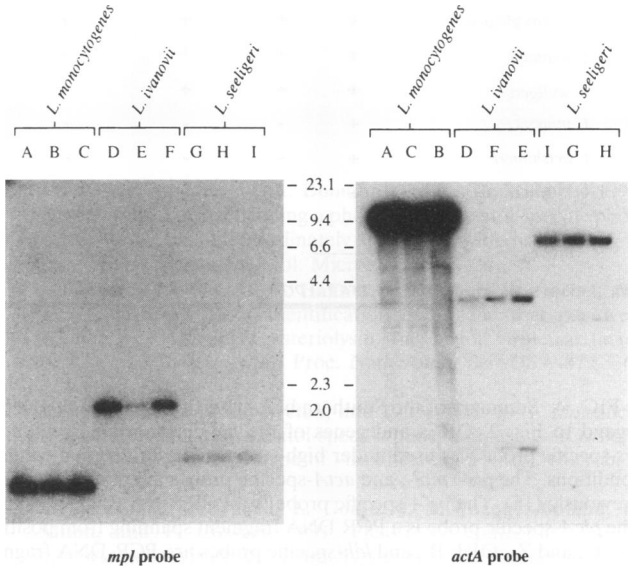

FIG. 3. Southern blots of DNAs from the six *Listeria* species hybridized under low-stringency conditions with an *mpl* probe (left) and an *actA* probe (right). Hybridization was carried out overnight at 37°C in a mixture of 30% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 10× Denhardt solution, and 200 μg of sonicated salmon DNA per ml. Filters were washed twice at 37°C in 1× SSC–0.1% sodium dodecyl sulfate (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Only the parts of the blots with hybridization signals (i.e., the part containing DNA from the hemolytic *Listeria* species) are shown. Lanes: A, *L. monocytogenes* LO28 (Bof 343); B, *L. monocytogenes* EGD (BUG 600); C, *L. monocytogenes* type strain SLCC 53^T (BUG 31); D, *L. ivanovii* type strain CIP7842^T (BUG 496); E, *L. ivanovii* CLIP 257 (BUG 497); F, *L. ivanovii* SLCC4121 (BUG 598); G, *L. seeligeri* type strain SLCC 3954^T (BUG 494); H, *L. seeligeri* CLIP 9529 (BUG 495); I, *L. seeligeri* SLCC3503 (BUG 599). All lanes contain the same amount of DNA, except lane E on the left panel and lanes D and F on the right panel which, from the ethidium bromide staining, contain less DNA. The *mpl*-specific probe is a PCR DNA fragment spanning from position 982 to position 1440 in the DNA sequence of *mpl* (28a). The *actA*-specific probe is a PCR DNA fragment spanning from position 958 to position 1510 in the DNA sequence of *actA* (38).

The function of the proteins encoded by *tms* and *ecourf1* is unknown. In contrast, it is well established that PRPP synthetases catalyze the formation of PRPP by the following reaction: ribose-5-phosphate + ATP → PRPP + AMP. Microorganisms contain about 10 enzymes which use PRPP as a substrate (17). These enzymes are constituents of the biosynthetic pathways leading to purine and pyrimidine nucleotides, histidine, tryptophan, and NAD.

The essential role of PRPP synthetase suggested that *prs* should be present in all *Listeria* species. By using a *prs*-specific probe for Southern hybridization under high-stringency conditions (Rapid Hybridization System; Amersham), we detected sequences homologous to the *L. monocytogenes prs* gene in *L. ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri*, and *Listeria grayi* (Fig. 2). This analysis thus revealed that *prs* delimits one end of the virulence gene cluster in *L. monocytogenes*. The other end had previously been identified downstream from the lecithinase operon (38). Indeed, downstream from the lecithinase operon, we had detected three ORFs: ORF A, ORF B, and *ldh*, an ORF homologous to genes encoding lactate dehydrogenases. We had shown that sequences homologous to ORF B and *ldh* could be detected under low-stringency conditions in all *Listeria* species (38). In *L. monocytogenes*, the virulence gene cluster is thus bordered by genes present in all *Listeria* species, *tms* and *prs* on one side and ORF A, ORF B, and *ldh* on the other side. It is 9.6 kb long and spans the *plcA-prfA* operon, *hly*, and the lecithinase operon (*mpl*, *actA*, and *plcB* genes and ORFs X, Y, and Z). We have searched for sequences at both ends of the virulence region which could be reminiscent of transposons or insertion sequence elements and would be suggestive of a block transfer of virulence genes, but no such elements were found.

L. ivanovii is the only other pathogenic species of the genus *Listeria*. It shares with *L. monocytogenes* important phenotypic characteristics. For example, it is hemolytic (13, 24, 37) and produces a phosphatidylinositol-specific phospholipase C (25,

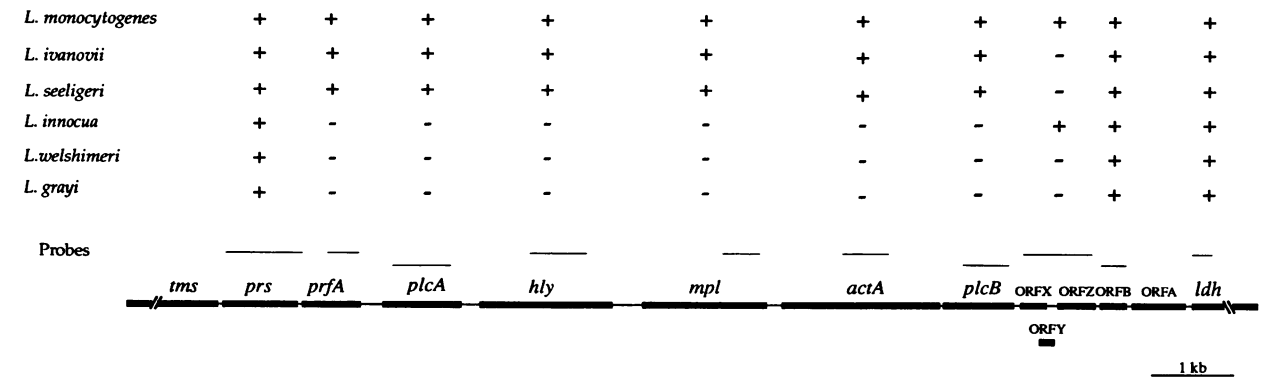

FIG. 4. Summary of the Southern blot hybridizations with the DNAs from the six *Listeria* species. The strains used were those described in the legend to Fig. 2. ORFs and genes of the *hly* chromosomal region are represented by thick lines; probes are represented by thin lines. The *prs*-specific probe was used under high-stringency hybridization conditions. All of the other probes were used under low-stringency hybridization conditions. The *prs*-, *mpl*-, and *actA*-specific probes are as described in the legends to Fig. 2 and 3. The *hly*-specific probe is probe 1 as described previously (15). The *prfA*-specific probe is a PCR DNA fragment spanning from position 321 to position 690 in the DNA sequence of *prfA* (28). The *plcA*-specific probe is a PCR DNA fragment spanning from position 431 to position 1074 in the DNA sequence of *plcA* (28b). The *plcB*-, ORF X-, Y-, and Z-, ORF B-, and *ldh*-specific probes are PCR DNA fragments spanning from positions 2625 to 2988, 3160 to 4023, 4109 to 4412, and 5259 to 5643, respectively, in the corresponding DNA sequence (38).

27). We were puzzled by our previous results (15, 38) and those of others (8) indicating that the region located downstream from *hly* was specific to *L. monocytogenes*. In these previous studies, the *mpl*-specific probe corresponded to the 5′ part of the *mpl* gene, and the *actA*-specific probe covered the entire *actA* gene. By using PCR, we designed a novel *mpl* probe, located in the 3′ part of the *mpl* gene and corresponding exactly to a region of the protein predicted to be important for activity and probably most conserved. This novel probe detected under low-stringency conditions of hybridization sequences similar to the *L. monocytogenes mpl* gene in *L. ivanovii* and *L. seeligeri* (Fig. 3) but not in the other nonhemolytic *Listeria* species (data not shown). By using a similar strategy and an internal *actA* probe, we detected sequences homologous to *actA* in *L. ivanovii* and *L. seeligeri* (Fig. 3). We finally extended our analysis by Southern blot hybridization to the whole virulence locus. The results are presented in Fig. 4. *prs* on one side and ORF B and *ldh* on the other side are present in all *Listeria* species. Characterized genes of the virulence gene cluster (i.e., *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*) are present only in the three hemolytic species, *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* and not in the three other nonhemolytic species, *L. innocua*, *L. grayi*, and *L. welshimeri*. The DNA region spanning ORFs X, Y, and Z is present only in *L. monocytogenes* and *L. innocua*, indicating that the border of the virulence gene cluster located downstream from *plcB* is not as clearly defined as the border located downstream from *prfA*. Interestingly, *L. innocua* is the species which is the most closely related to *L. monocytogenes* (33).

In conclusion, the results presented in this article, together with previous data (38), indicate that the virulence gene cluster of *L. monocytogenes* is present in the other pathogenic species *L. ivanovii* and in the nonpathogenic but weakly hemolytic species *L. seeligeri*. Concerning *L. seeligeri*, from the very low level of expression of its hemolysin (13) and the absence of phosphatidylinositol-specific phospholipase C activity (25, 27) and lecithinase (14), one can anticipate that this species is nonpathogenic because of a down-regulation of most virulence genes. In the case of *L. ivanovii*, it is in fact not surprising that the region located downstream from *hly* is present in this species, since it is well established that the cellular behavior of *L. ivanovii* is quite similar to that of *L. monocytogenes*. Indeed, this species escapes from the vacuole, induces actin assembly, and spreads from cell to cell (reference 21 and our unpublished results). Recent results from Southern blot hybridization, restriction mapping, and sequence analysis indicate that, in *L. ivanovii*, the gene organization from *prfA* up to *plcB* is identical to that of *L. monocytogenes* (reference 15 and our unpublished results). In view of the present strong interest in the actin-based motility of *L. monocytogenes* and in the role of *actA* and its ligands in this process (6), it is clear that, among future issues of the present study, the structure of the *actA* gene of *L. ivanovii* identified here should be very informative and help to identify those regions of the ActA protein which are relevant for its activity.

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